SciMedCentral

JSM Biotechnology & Biomedical Engineering

Research Article

In vitro and *In vivo* Antibacterial Activity of Marine Alga *Turbinaria ornata* against *Pseudomonas aeruginosa* in the Freshwater Prawn *Macrobrachium rosenbergii*

Gopalan Rajkumar[1], Periyakali Saravana Bhavan[1]*, Veeran Srinivasan[2], Annamalai Asaikutti[1], and Rajendran Udayasuriyan[1]

[1]*Department of Zoology, Bharathiar University, India*
[2]*Department of Animal Science, Bharathidasan University, India*

***Corresponding author**
Periyakali Saravana Bhavan, Department of Zoology, Bharathiar University, Coimbatore - 641046, Tamil Nadu, India, Tel: 91-422-2428495/91-9842498138; Email: bhavan@buc.edu.in

Submitted: 26 September 2017

Accepted: 13 October 2017

Published: 16 October 2017

ISSN: 2333-7117

Keywords
- *T. ornata*
- *M. rosenbergii*
- *P. aeruginosa*
- Survival
- Brown spot
- Hepatopancreas
- Protein

Abstract

The *in vitro* and *in vivo* antibacterial activity of marine alga, *Turbinaria ornata* against *Pseudomonas aeruginosa* on *Macrobrachium rosenbergii* was studied. The hexanic, acetonic and methanolic extracts of *T. ornata* were subjected to *in vitro* study, which showed that the methanolic extract of *T. ornata* was worked well and produced 24.02 ± 0.47mm zone of inhibition against *P. aeruginosa*. In the *in vivo* study, the characteristic appearance of brown/black spots was detected all over the body of *M. rosenbergii* when the prawns were subjected to immersion in water containing *P. aeruginosa* (10^7 cells/mL) and fed with basal diet contained no incorporated *T. ornata*. Activities of metabolic enzymes (GOT and GPT) and antioxidant enzymes (SOD and catalase), and lipid peroxidation (LPO) were found to be significantly increased in the hepatopancreas of *M. rosenbergii* due to the infection with *P. aeruginosa*. These effects were greatly reduced/ neutralized when *M. rosenbergii* fed with methanolic extract of *T. ornata* incorporated feed, which in turn ultimately enhanced the survival rate of *M. rosenbergii* against *P. aeruginosa* infection. The 2D gel electrophoresis revealed degradation of hepatopancreatic proteins in *P. aeruginosa* infected *M. rosenbergii*, whereas the reverse was seen in methanolic extracts of *T. ornata* incorporated feed fed prawns. Thus, *T. ornata* possessed the potency of anti *P. aeruginosa*. One of the active principles of *T. ornata*, 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]] was found to be bind with the protein, exotoxin-A of *P. aeruginosa* when molecular docking was performed. Therefore, there is scope for developing aquaculture medicine with *T. ornata* against *Pseudomonas* infection.

ABBREVIATIONS

PL: Post Larvae; SOD: Superoxide Dismutase; CAT: Catalase; GOT: Glutamic Oxaloacetic Transaminase; GPT: Glutamic Pyruvic Transaminase; LPO: Lipid Peroxidation; 2D gel: 2 Dimensional Gel Electrophoresis

INTRODUCTION

Aquaculture has improved the socio-economic status of rural people [1]. The freshwater prawn genus *Macrobrachium* (Palaemonidae), distributed throughout the tropical and subtropical zones of the world. Worldwide, 100 species of freshwater prawns have been recorded, among them 40 species are found in India [2]. *Macrobrachium rosenbergii* is a potential candidate species for aquaculture, because it provides nutritious delicacy for human being, and fetched for good price with high demand in both domestic and export markets [3]. Disease outbreak due to viral, bacterial and fungal infections threatened the sustainability and profitability of crustacean aquaculture, which has become an issue of global concern [4,5]. The bacterial disease associated with *Aeromonas* and other genera of chitinolytic bacteria (*Pseudomonas, Vibrio, Beneckea* and *Leucothrix*) leaves brown/black spots, necrosis in the hepatopancreas and gill disablement [6,7]. A pathogenic Gram negative bacterium, *Pseudomonas aeruginosa* can be found in water, soil and sediments [8,9]. Therefore, incidence of *P. aeruginosa* infections in fish and shellfish including freshwater prawn *M. rosenbergii* have been reported [10-13]. Its control is a challenging task.

About 6000 species of different marine algae have been reported under green, brown and red categories. They are used as food, feed and fertilizer. They have biomedical potentials due to presence of many bioactive principles as anti-inflammatory, anti-microbial, anti-viral and anti-tumoral agent [14]. Therefore, the present study aimed to examine the antibacterial activity of the brown alga *Turbinaria ornata* by their *in vitro* (by using hexanic, acetonic and methanolic extracts) and *in vivo* activity against *Pseudomonas aeruginosa* induced infection in *M. rosenbergii* PL, offering 1.5% methanolic extract of *T. ornata*

incorporated diet. In order to reveal this, morphological changes, activities of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] and metabolic enzymes [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)], lipid peroxidation (LPO), 2D-gel pattern of hepatopancreas, and molecular docking of the ligand, 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]] (one of the active principle compound of *T. ornata*) with the protein, exotoxin-A of *P. aeruginosa* were studied.

MATERIALS AND METHODS

Collection and identification of marine alga

The marine alga *Turbinaria ornata* was collected from the intertidal region of Mandapam coast (Lat. 9°17'N; Lon. 79°19'E) of Gulf of Mannar, south-east coast of Tamil Nadu, India. The macroalga species was identified based on its morphology by using identification manual of "Economically Important Seaweeds" [15] published by Central Marine Fisheries Research Institute (ICAR), Kochi, India. Finally, the species was authenticated by Botanical Survey of India (BSI), Coimbatore, India.

Preparation of extracts of *T. ornata*

The collected sample was cleaned well with seawater to remove all the extraneous matter such as epiphytes, sand particles and necrotic parts, and brought to the laboratory in plastic bags. The sample was then thoroughly washed with freshwater, blotted, spread out and shade dried at room temperature for 2 weeks, and was ground to fine powder.

The powdered sample of *T. ornata* (75g) was separately packed in Whatmann No. 1 filter paper and Soxhlet extraction was done with 450ml (1:6 w/v) of hexane, acetone and methanol individually for 9 h each (36 cycle) until a clear colorless solution was obtained. These extracts were filtered by using double layer muslin cloth, concentrated at 40-50°C using rotary vacuum evaporator (ROTAVAP) attached with ultra-cryostat and dried at 40°C under hot air oven [16]. The dark, gummy solid obtained was used for further investigation.

Determination of *in vitro* antibacterial activity

The *in-vitro* antibacterial activity was studied by agar well diffusion method [17]. To activate the bacterium before inoculation, it was cultured on nutrient broth (Himedia, INDIA) and incubated at 37°C for 24 h. For inoculums preparation and antibacterial assay approximately, 20ml of the autoclaved medium Mueller-Hinton agar (Himedia, INDIA) was dispensed into sterile plates and allowed to solidify under aseptic conditions. Then, the bacterium *Pseudomonas aeruginosa* was inoculated and spread with a sterile swab on the surface of agar plates and three wells of 5mm diameter were aseptically made on each plate. The crude extracts of *T. ornata* were reconstituted in respective solvents at a concentration of 250mg/mL. The wells were then filled with 50µl of each solvent extracts of *T. ornata*. The individual solvents (hexane, acetone and methanol) and amoxicillin (100mg/ml) were used as negative and positive controls respectively. The plates were incubated overnight at 37°C. The diameter of the zones of inhibition was measured and compared to that of positive and negative controls. The experiment was conducted three times.

Experimental feeds

The following branded feed basal ingredients (BI) were used to formulate the experimental feed. For protein sources, fish meal (25%), groundnut oilcake (25%) and soybean meal (25%) were taken. Wheat bran (10%) was used as carbohydrate source. For lipid source, Sunflower oil (2%) was taken. Tapioca flour (5%) and egg albumin (7%) were used as binding agents. The powdered basal ingredients, such as fish meal, groundnut oilcake, soy bean meal, wheat bran were thoroughly mixed. Then sterilized water was used to prepare the dough, which was steam cooked and cooled at room temperature. Then Sunflower oil was added with the dough and mixed well. Then, methanolic extract of *T. ornata* was separately incorporated with the dough at concentration of 1.5% and mixed well. Then tapioca flour and egg albumin were added and mixed well. Finally, 1% of vitamin B-complex forte with vitamin C (BECOSULES® CAPSULES, Pfizer Ltd., Navi Mumbai, India) and a pinch of salt were also added and thoroughly mixed. Sterilized water was adequately added for maintaining the mixer in moist and paste form. This was pelletized in a manual pelletizer fixed with 3mm diameter mesh. The pellets were immediately dried in a thermostatic oven at 37-40°C for one hour to quickly reduce the moisture in order to keep them intact, and then shade dried until they reached constant weight. To maintain its brittleness and prevent fungal attack they were kept in air tight jars and stored at -20°C, and used afresh. The proximate composition of organic matters present in the basal diet formulated was determined by adopting the methodology of Castell and Tiews [18] as given in AOAC [19] manual, which contains 40.5% crude protein, 5.6% crude fat, 3.4% crude fibre, 9% total ash, 8.6 % moisture, 32.9% carbohydrate (total nitrogen free extract) and gross energy, 4281kcal/kg.

Experimental animal

The post larvae (PL-10) of the freshwater prawn, *Macrobrachium rosenbergii* were procured from ADAK Hatchery, Odayam, Varkala, Thiruvananthapuram, Kerala, India. They were transported to the laboratory in polythene bags filled with oxygenated water and acclimatized to ambient laboratory conditions for 2 weeks in cement tank (6 × 3 × 3 feet) with ground water (temperature, 27 ± 1.0; pH, 7 ± 0.15; total dissolved solids, 0.9 ± 0.005g L^{-1}; dissolved oxygen, 7.2 ± 0.55mg L^{-1}; BOD 30.0 ± 1.30mg L^{-1}; COD, 125.0 ± 3.2mg L^{-1}; ammonia, 0.028 ± 0.004mg L^{-1}). During acclimatization the prawns were fed with boiled egg albumin and artificially formulate feed of our own. More than 50% of tank water was routinely changed every day in order to maintain a healthy environment and adequate aeration was also provided to ensure sufficient oxygen supply to the prawns and an environment devoid of accumulated metabolic wastes. The unfed feeds, faeces, moult and dead prawns if any were removed by siphoning without disturbing the prawns.

Feeding trial

Two groups of PL-30 staged prawns (1.45 ± 0.24cm and 0.10 ± 0.03g) were taken in triplicate experimental set-up. Each group consisted of 30 PL in an aquarium maintained with 30 L of ground water. The group-I served as control and fed with feed formulated by using BI only. The group-II was fed with 1.5% of methanolic extract of *T. ornata* incorporated diet. The water medium was renewed every 24 h by siphoning method without severe disturbance to the PL and aerated adequately. The test prawns were fed with above prepared diets at 10% of

body weight twice a day (8.00 a.m. and 8.00 p.m.) consecutively for 60 days and assumed that they have reached PL-90. During the feeding trial, the unfed feed, feces and moults if any were removed on a daily basis while renewing the aquarium water. The similar experimental set-up was maintained then and there to study different parameters.

Pathogen challenge test

The pathogenic bacterium, *P. aeruginosa* isolated and characterized previously in our laboratory [20] from infected (brown/ black spotted) *M. rosenbergii* taken from the wild (GenBank accession number, KX398049) was used in this study for immersion assay. Culture of *P. aeruginosa* was maintained using nutrient agar. A preliminary bioassay was conducted to assess the survival capacity of PL in 1.5ml L^{-1} cultured broth [21]. Actually, PL was immersed for 2, 4, 6 h duration and allowed to survive in normal water for 10 days (survival rate, SR = No. of live prawns/ No. of prawns introduced × 100). This revealed that no mortality was occurred in any of the immersion duration. Therefore, the concentrations were magnified to 10^1, 10^3, 10^5 and 10^7 cells mL^{-1} and the mortality was assessed against 2, 4, 6 h immersion durations. Finally, 10^7 cells mL^{-1} concentration was chosen under 2h immersion duration.

The above experimental PL group-II that was in feeding trial for 60 days subjected to immersion assay (10^7 cells mL^{-1}) for 2 h was extended as challenged treatment group for further 21 days, non-infected freshwater medium was used for renewal every day and they were continuously fed with 1.5% of methanolic extract of *T. ornata* incorporated diet. The control (group-I) was divided into two sub-groups, one negative control and one positive control (immersed/ infected control), simultaneous and separately maintained for 21 days, the water was renewed and feeding was allowed as mentioned earlier with BI. Actually, the group-I (control PL) was dived into unchallenged and challenged controls. These prawns were observed for morphological changes, appearance of brown/black spots.

Activities of metabolic enzymes

The metabolic enzymes such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were analyzed by the method of Reitman and Frankel [22] using a med source kit (Medsource Ozone Biomedicals Pvt. Ltd. Haryana, India). Each 100mg of hepatopancreas tissues were homogenized in 0.25M sucrose and centrifuged at 3300g for 20 min in a high speed cooling centrifuge at 4°C. The supernatant was used as the enzyme source.

GOT analysis, the substrate solution, L-aspartic acid (500µL; pH, 7.4) was added to a 100µL sample and incubated at 37°C for 1 h. Further, 500µL of 2, 4-dinitrophenyl hydrazine was added and allowed to stand for 20 min at room temperature. Then 3mL of freshly prepared 4 N sodium hydroxide solution was added to the above solutions. The color development was read at 505nm using spectrophotometer within 15 min. Sodium pyruvate (160 U/L^{-1}) was used as a calibrator. The activity of GOT was expressed as Unit L^{-1}.

GPT analysis, buffered L-alanine, 2-oxoglutarate substrate (500µL; pH, 7.4) was added to a 100µL sample and incubated at 37°C for 20 min. With this, 500µL of 2, 4-dinitrophenyl hydrazine was added and allowed to stand at room temperature for 30 min followed by the addition of 3mL of freshly prepared 4 N sodium hydroxide solution. The color development was read at 505nm using a spectrophotometer within 15 min. Sodium pyruvate (170 $U\ L^{-1}$) was used as a calibrator. The activity of GPT was expressed as Unit L^{-1}.

Activities of enzymatic antioxidants and lipid peroxidation

Tissues of hepatopancreas (100mg each) was homogenized (10% w/v) in ice-cold 50mM Tris buffer (pH 7.4), centrifuged at 9329g for 20 min at 4°C and the supernatant was used to assay the activities superoxide dismutase (SOD) and catalase (CAT).

SOD activity was measured by pyrogallol (10mM) autoxidation in Tris buffer (50mM, pH 7.0) by adopting the method of Marklund and Marklund [23]. The reaction was initiated by the addition of NADH. The mixture was incubated at 30°C for 90 sec and arrested by the addition of glacial acetic acid. The reaction mixture was then shaken with n-butanol and the intensity of the chromogen in the butanol layer was measured at 560nm using spectrophotometer. The specific activity of the enzyme was expressed in unit/ mg protein.

CAT activity was measured by using H_2O_2 as the substrate in phosphate buffer by adopting the method of Sinha [24]. The reaction was initiated by the addition of phosphate buffer (0.01 M, pH 7.1), H_2O_2 (0.2 M). After 60 sec the reaction was stopped by the addition of dichromate acetic acid reagent. The absorbance of the chromophore was read at 620nm. The activity of CAT was expressed as µM of hydrogen peroxide consumed/ minute/ mg protein.

Lipid peroxidation (LPO) in tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA) by adopting the method of Ohkawa et al. [25]. The absorbance of the supernatant was measured at 535nm against the reagent blank. TBARS was expressed as nM of malondialdehyde (MDA)/ mg protein. Concentration of soluble proteins was determined by the method of Lowry et al. [26].

Profiles of proteins

After 21 days immersion assay, the SDS-PAGE analysis was done on the prawns of unchallenged control, challenged control, and challenged treatment groups. The hepatopancreas tissue sample was homogenized in phosphate buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4, pH-7.4) under ice cooled condition and centrifuged at 1500rpm at 4°C for 5 min. The soluble protein content in supernatant was determined by Bradford [27]. Bradford reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml of 95% ethanol and 100ml of 85% phosphoric acid. The mixture was then topped to 1 L with deionized water and stirred overnight. The final mixture was filtered and stored in room temperature away from light. SDS-PAGE was performed [28] on vertical slab gel with 4% stacking and 10% separating gels. Marker consisting of six different molecular weight proteins (Medox-Biotech Pvt. Ltd., India), such as116kDa (β-galactosidase), 66kDa (bovine serum albumin), 45kDa (ovalbumin), 29kDa (carbonic anhydrase), 20kDa (soybean trypsin inhibitor) and 14kDa (lysozyme) were also ran simultaneously. The polypeptides banding patterns between control and test prawns were compared by using information on apparent molecular masses of bands and their intensity.

2D Gel electrophoresis (pH dependent and anionic exchange)

After 21 days immersion assay, 2D Gel analysis was done on *M. rosenbergii* of negative control, positive control, and challenged prawns fed with 1.5% of methanolic extracts of *T. ornata* incorporated feed. The hepatopancreas was aseptically removed and extracted with 1% triton X-100 buffer [1% triton X-100, 20mM Tris-HCl (pH -8.0), 150mM NaCl and 10µl protease inhibitor cocktail]. The soluble protein concentration in the hepatopancreas was estimated and quantified [27].

Rehydration of the dry strips was done in rehydration solution at 250µl per strip in the presence of 8 M urea, 2% v/v CHAPS, trace of bromophenol blue, 50mM DTT, 2.5µl IPG buffer and dry-strip cover liquid. The first dimension of 2-DE was performed in IPGphor IEF system. A total of 250µg of proteins from each hepatopancreas samples were loaded onto each IPG strip (nonlinear pH 3-10; 13cm long). The focused IPG strip was incubated for 15 min in an equilibration buffer containing 87% w/v glycerol, 60.06 Mw Urea, 2% w/v SDS, 50mM Tris-HCl (pH 8.8), 100mM DTT, and trace of bromophenol blue. Before running the second dimension the strip was then further equilibrated for 15 min in a similar buffer, which replaced 100mM DTT with 250mM of iodoacetamide. The IPG strip was placed onto the top of 12% SDS-PAGE gel and sealed with hot agarose (1% w/v). After the run (up to 8 hr at 100 V DC under air conditioned room), the gel was fixed in a fixative solution containing 500ml ethanol and 120ml acetic acid, 500µl formaldehyde 35%, 380ml dH_2O and stained with Coomassie Brilliant Blue G-250 overnight, then de-stained with solution containing methanol (43%), acetic acid (7ml) and double distilled water (50ml) until a clear background was achieved. All the images were collected on Bio-Red images (Bio-Red Laboratories, Inc.).

Molecular docking analysis

Intermediary steps, such as PDBQT files for protein (bacterial toxin) and ligands (bioactive compound identified) preparation and grid box creation were done by using graphical user interface program AutoDock Tools (ADT). ADT assigned polar hydrogens, united atom Kollman charges, solvation parameters and fragmental volumes to the protein. AutoDock saved the prepared file in PDBQT format. Auto Grid was used for the preparation of the grid map using a grid box. The grid size was set to 60 × 60 × 60 xyz points with grid spacing of 0.375 Å and grid center was designated at dimensions (x, -1.095; y, -1.554 and z, 3.894). A scoring grid is calculated from the ligand structure to minimize the computation time.

AutoDock was employed for docking using protein and ligand information along with grid box properties in the configuration file. AutoDock employs iterated local search global optimizer [29,30]. During the docking procedure, both the protein and ligands are considered as rigid. The results less than 1.0 Å in positional root-mean square deviation (RMSD) was clustered together and represented by the result with the most favorable free energy of binding. The pose with lowest energy of binding or binding affinity was extracted and aligned with receptor structure for further analysis.

STATISTICAL ANALYSIS

The data were expressed as mean ± SD (n=3), and analyzed by one-way analysis of variance (ANOVA) using SPSS (version-20), and subsequent post hoc multiple comparison, Duncan's multiple range test (DMRT) to compare the significant differences among treatments at $P<0.05$.

RESULTS

In-vitro antibacterial activity of *T. ornata*

Hexanic, acetonic and methanolic extracts of *T. ornata* showed antibacterial activity against *P. aeruginosa* (Figure 1; Table 1). This was assessed by comparing the zone of inhibition of standard antibiotic, amoxicillin. The inhibition zone of 19mm, 23mm and 24mm was observed for hexanic, acetonic and methanolic extracts, respectively. The positive control (amoxicillin) produced 21-24mm zone of inhibition. The negative controls (hexane, acetone and methanol) did not show any inhibition.

Survival rate and morphology of *P. aeruginosa* challenged prawn

The survival rate of the uninfected control was higher when compared with infected control. When the infection due to *P. aeruginosa* was challenged with 1.5% of methanolic extract of *T. ornata* incorporated feed, the PL showed higher survival rate than that of the uninfected control. Therefore, *T. ornata* has enhanced the survival rate in *M. rosenbergii* under infection by *P. aeruginosa* (Table 2). The positive control showed external sign of infection, such as brown/black spots over the body, ruptured uropods and loss of periopods (Figure 2). These signs were not seen in the uninfected control, nor in the prawns fed with 1.5% of methanolic extract of *T. ornata* incorporated feed.

Activities of metabolic enzymes, antioxidant and lipid peroxidation

The activities of GOT, GPT, SOD, CAT and LPO were significantly increased in *P. aeruginosa* infected control ($P<0.05$) when compared to uninfected control and the prawns fed with 1.5% of methanolic extracts of *T. ornata* incorporated feed (Table 3), indicating that this marine algae have the capacity to neutralize the effect of the pathogenic bacterium, *P. aeruginosa*.

Profiles of proteins

Polypeptide bands between 139-12kDa were resolved in the hepatopancreas of *M. rosenbergii* PL (Figure 3). In the uninfected control, 14 out of 18 protein bands, were prominently stained (139, 116, 59, 50, 47, 45, 39, 36, 29, 20, 17, 16, 14 and 12kDa). The infected control presented, weaker stain intensity and 139, 116 47, 20, 16 and 12kDa protein bands were less visible. *P. aeruginosa* infection challenged with 1.5% methanolic extract of *T. ornata* incorporated feeds fed prawns showed several protein bands with high, the stain intensity, such as 139, 116, 66, 61, 59, 56, 50, 47, 45, 32, 29, 20, 19, 17, 16, 14 and 12kDa.

2D Gel electrophoresis

The hepatopancreas of all three categories of prawns showed, 24 marked protein spots (Figure 4). All the marked spots were prominent and clear in the uninfected control (Figure 4a), whereas in the *P. aeruginosa* infected control, all these protein spots were correspondingly dull and dispersed (Figure 4b). In *T. ornata* incorporated feeds fed prawns, all these protein spots were found to be very prominent and clear, similarly to the uninfected control (Figure 4c).

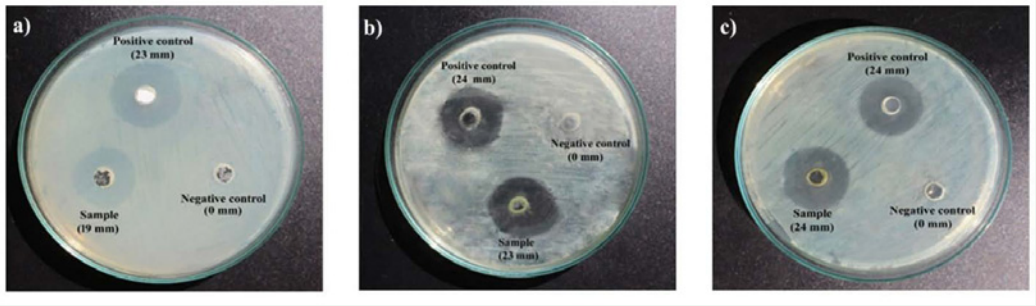

Figure 1 *In-vitro* antibacterial activity of *T. ornata* against *P. aeruginos*: a) Hexanic extract; b) Acetonic extract; c) Methanolic extract.

Table 1: *In-vitro* antibacterial activity of *T. ornata* extracts against *P. aeruginosa.*

Extracts concentrations	Zone of inhibition (mm)
Positive control, Amoxicillin (5mg or 50µl/ well = 100mg/ml	23.38 ± 1.04
Negative controls (Hexane, acetone and methanol: 50µl/ well)	--
Hexanic extract (12.5mg or 50µl/ well = 250mg/ml)	19.01 ± 0.46
Acetonic extract (12.5mg or 50µl/ well = 250mg/ml)	22.52 ± 0.58
Methanolic extract (12.5mg or 50µl/ well = 250mg/ml)	24.02 ± 0.47

Each value represents mean ± SD; *n*=3 ('*n*' for positive control =18). Mean values within the same column sharing the different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).

Molecular docking

In our previous study, five bioactive compounds from *T. ornata* were recorded: 4,8,12,16-Octadecatetraen-1-ol, 4,9,13,17-tetramethyl; Neophytadiene; 17 Pentatriacontene; 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]; and Squalene [16]. All of these compounds were taken as ligands and subjected to molecular docking with the protein, exotoxin-A from *P. aeruginosa*. Among the five compounds, the exotoxin-A was found to be bind with only one bio-active compound, 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]. The gliding score, docking score and length of the H bond were shown in tables 4 and 5, and figure 5. The ligand, 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]] was found to be bind with active site of GLY 441 (amino acid of protein, exotoxin-A) and the bond length was 2.182 for hydrogen to oxygen from protein and 2.147 for sites of ligand from oxygen to hydrogen, respectively.

DISCUSSION

In the present study, methanolic extracts of *T. ornata* showed anti-bacterial activity against *P. aeruginosa.* Bioactive components present in seaweeds inhibit the growth of some gram-positive and gram-negative bacteria [31]. Marine algal extracts have been used as curative and preventive agents against helminths, bacteria, virus, fungi, inflammation, cough, hypertension, tumor and diarrhoea [31-34]. Pholdaeng and Pongsamart [35] reported that *Penaeus monodon* pre-fed with

Figure 2 Appearance black/brown spots on *M. rosenbergii* after challenging study: a) Negative control, looked normal in color; b) and c) Dorsal and lateral views of positive control, infected with *P. aeruginosa*, which exhibited brown/black spots over the body; d) Absence of brown/black spots on the body of *M. rosenbergii* fed with 1.5% methanolic extracts of *T. ornata,* which showed resistance to *P. aeruginosa* infection .

Durio zibethinus supplemented diet showed higher resistance against viral and bacterial pathogens. Holdt and Kraan [36] have reported that polysaccharides (alginate, fucoidan and laminarin) present in algal extracts can control viral and bacterial infections.

In the present study, methanolic extract of *T. ornata* incorporated diet improved survival performance in *M. rosenbergii* PL. Immanuel, et al. [37], demonstrated that herbal and algal extracts may be effectively used as a dietary source to enhance the disease resistance as well as to increase survival and production of *Penaeus indicus* in aquaculture systems. Manilal et al. [38], contemplated the effect of *Acrosiphonia orientalis* incorporated feed on the survival rate of *P. monodon* against *Vibrio harveyi* and *Vibrio alginolyticus* infections. Similarly, *Ulva* diet at a dose of 1000mg/kg has enhanced the survival rate of *P.*

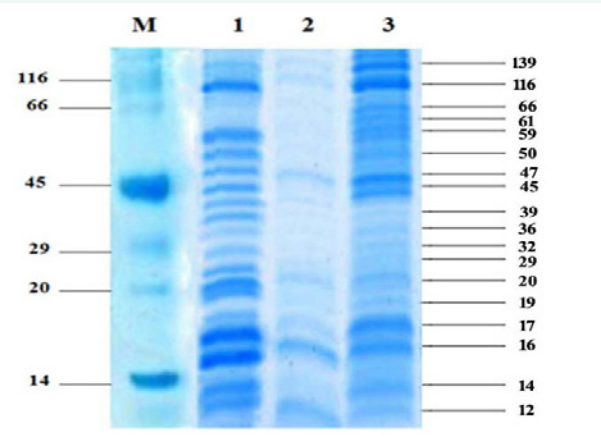

Figure 3 SDS-PAGE (12%) pattern of hepatopancreatic proteins of *M. rosenbergii*: M - Marker; Lane-1 - Negative control (uninfected); Lane-2 - Positive control (infected by *P. aeruginosa*); Lane-3 - *P. aeruginosa* infection challenged with methanolic extract of *T. ornata* (1.5%) incorporated feed.

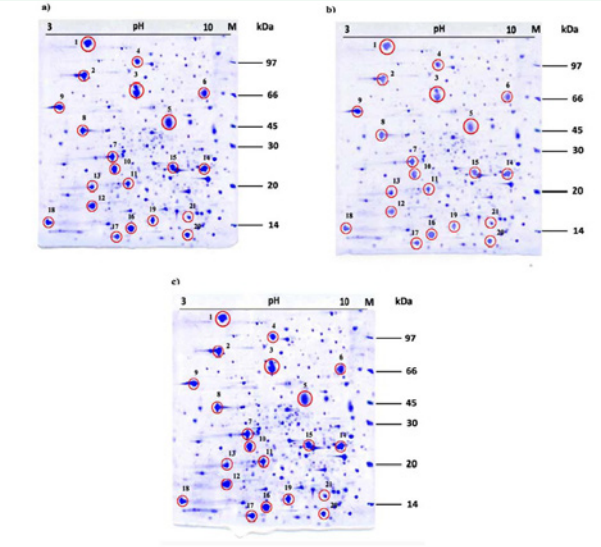

Figure 4 2D gel pattern of hepatopancreatic protein of *M. rosenbergii* (I-dimension: pH dependent; II-dimension: anionic exchange): a) Negative control (uninfected); b) Positive control (infected by *P. aeruginosa*); c) *P. aeruginosa* infection challenged with methanolic extract of *T. ornata* (1.5%) incorporated feed.

Figure 5 3D docking structure of 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R,R-(E)]] of *T. ornata* with exotoxin-A of *P. aeruginosa*: Benzine ring, appeared with green color, is the target protein, exotoxin-A of *P. aeruginosa*.

Table 2: Survival rate of *M. rosenbergii* PL against *P. aeruginosa* infection treated with methanolic extracts of *T. ornata* incorporated feed.

Time (Days)	Negative control (uninfected)	Positive control (infected)	*T. ornata* (1.5%)
0	100.00 ± 0.00 [a]	100.00 ± 0.00 [a]	100.00 ± 0.00 [a]
7	100.00 ± 0.00 [a]	97.77 ± 1.92 [a]	100.00 ± 0.00 [a]
14	95.55 ± 1.92 [abc]	75.55 ± 1.92 [d]	96.66 ± 3.33 [ab]
21	81.11 ± 1.92 [c]	61.11 ± 1.92 [d]	92.22 ± 1.92 [b]

Each value represents mean ± SD; *n*=3; Mean values within the same row sharing different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).

Table 3: Activities of antioxidant and metabolic enzymes, and lipid peroxidation in the hepatopancreas of *M. rosenbergii* PL on 21st day after challenged with *P. aeruginosa* infection treated with methanolic extract of *T. ornata* incorporated feed.

Parameters	Negative control (uninfected)	Positive control (infected)	*T. ornata* (1.5%)
SOD (µmol /min/mg protein)	12.56 ± 1.24[b]	26.76 ± 2.62[a]	14.88 ± 1.93[b]
CAT (Unit/mg protein)	22.56 ± 2.47[b]	39.28 ± 3.81[a]	24.28 ± 2.74[b]
LPO (nmol MDA/mg protein)	1.70 ± 0.08[b]	8.65 ± 0.31[a]	2.34 ± 0.28[b]
GOT (Unit/L)	12.17 ± 1.48[b]	22.83 ± 1.74[a]	14.23 ± 1.58[b]
GPT (Unit/L)	12.79 ± 1.38[b]	25.43 ± 2.19[a]	14.46 ± 1.52[b]

Each value represents mean ± SD; *n*=3; Mean values within the same row sharing different alphabetical superscripts are statistically significant at $P < 0.05$ (one way ANOVA and subsequent *post hoc* multiple comparison with DMRT).
SOD: Superoxide Dismutase; CAT: Catalase; LPO: Lipid Peroxidation; MDA: Malondialdehyde; GOT: Glutamic Oxaloacetic Transaminase; GPT: Glutamic Pyruvic Transaminase.

monodon against *Vibrio* infection [39].

Bhavan and Geraldine [40] reported that hepatopancreas is a sensitive organ predisposed to injury by water born diseases and pollutants. Brown and black spot disease caused by *P. aeruginosa* infection led to pale hepatopancreas, which affect the biosynthesis of proteins, and could have resulted in loss of cellular ions and proteins. Therefore, breakdown/degradation of proteins may have occurred as a physiological stress response in prawns infected with *P. aeruginosa*. The breakdown of protein might have overcomed its synthesis. Thus, the stain intensity of various polypeptide bands of the hepatopancreas of *M. rosenbergii* infected with *P. aeruginosa* was considerably reduced. Protein degradation may occur due to excessive proteolysis to overcome the metabolic stress, as deposited protein in the cytoplasm can easily be used to replace the loss of proteins that occur during physiological stress [41,42].

In this study, the expression of proteins and their alterations were observed in 24 protein spots in the hepatopancreas of *M. rosenbergii* infected with *P. aerugionsa*. Somboonwiwat, et al. [43], reported that a total of 27 different protein spots were expressed in hemolymph of *P. monodon* infected with *Vibrio*

Table 4: Details of glide score, docking score and hydrogen bond length for ligand and exotoxin-A protein.

Name of compound	Glide score	Dock score	H Bond	Potential energy	Binding energy
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]	-3.0	-3.0	-4.11	125.432	-57.387

Table 5: Complex of bond length of amino acids for protein and ligand.

Name of compound	AA from protein	AA from ligand	Interacting residue	Bond Length
2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]	Hydrogen	Oxygen	GLY 441	2.182
	Oxygen	Hydrogen	GLY 441	2.147

AA: Amino Acid; GLY: Glycine

harveyi. Alinejad, et al. [44], reported that an average 20 out of 300 protein spots in each prawn hemocyte gel underwent considerable alterations in their expression levels upon infectious hypodermal and hematopoietic necrosis virus (IHHNV) infection in *M. rosenbergii.*

When *P. aeruginosa*, exotoxin-A was targeted, it exerted cellular toxicity through ADP ribosylation of translation elongation factor 2, which resulted into enzyme cleavage activity and binding of cell surface receptor and thus, caused toxicity in infected cells [45]. In this study, the active principle compound of *T. ornata* [2-Hexadecen-1-ol,3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]] has shown great anti-bacterial activity against exotoxin-A bio-informatically by binding and interacting. This has to be further studied from a pharmaceutical point of view after isolation, purification and characterization of this compound.

CONCLUSION

The methanolic extract of *T. ornata* has demonstrated potential to neutralize the effects occurred in *M. rosenbergii* due to *P. aeruginosa* infection. The significant increase in GOT and GPT in positive control indicated the fact that *P. aeruginosa* causes hepatotoxicity in *M. rosenbergii*, therefore, the LPO was operational, which in turn generate oxygen free radicals, and thus, SOD and catalase were activated, which in turn ultimately reflected on the hepatopancreatic protein pattern. Thus, all the marked 24 protein spots were dull due to their degradation. The unaltered GOT, GPT, LPO, SOD and CAT mechanism in *M. rosenbergii* fed with methanolic extract of *T. ornata* incorporated feed indicated that the algae has possessed the capacity to neutralize/ resist the effects of *P. aeruginosa* infection. This may be due to the active principle compound [2-Hexadecen-1-ol,3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]] present in the algae, which ultimately enhanced the survival rate of *M. rosenbergii* against *P. aeruginosa* infection. Moreover, all the 24 marked protein spots were very clear in *M. rosenbergii* fed with methanolic extract of *T. ornata* incorporated feed. Therefore, there are scopes for developing aquaculture medicine with *T. ornata* against *Pseudomonas* infection.

ACKNOWLEDGEMENTS

The authors are gratefully acknowledged Dr. M. Palanisamy, Scientist 'C', Southern Regional Centre, Botanical Survey of India (BSI), Coimbatore, India, for authentication of the marine alga, *T. ornata*.

REFERENCES

1. http://www.fao.org/fishery/statistics/en.
2. Mariappan P, Balamurugan P, Balasundaram C. Freshwater prawn, *Macrobrachium nobilii*, a promising candidate for rural nutrition. Aquacult Asia. 2003; 8: 13-14.
3. Radheshyam DR. Farming the freshwater prawn (*Macrobrachium malcolmsonii*), Aquaculture Asia Magazine, January-March 2009: 29-32.
4. Afsharnasab M, Mortezaei R, Yegane V, Kazemi B. Gross sign, histopathology and polymerase chain reaction observations of white spot syndrome virus in shrimp specific pathogen free *Litopeneaus vannamei* in Iran. Asian J Anim Vet Adv. 2009; 4: 297-305.
5. Fouzi MNM, Shariff M, Yusoff FM. Stress quantification in *Penaeus monodon* exposed to different levels of ammonia and subsequent infection to WSSV. Res J Vet Sci. 2012; 5: 12-24.
6. Lombardi JV, Labao VL. Disease and conditioning factors of mortality in larval culture of prawn of the genus *Macrobrachium*. In Proceedings of the 3rd Brazilian Symposium on Shrimp Culture. Joao Pessoa, Paraiba, Brazil, 1991a; 401-408.
7. Lombardi JV, Labao VL. Diseases and other factors leading to mortality in juveniles and adults belonging to the genus *Macrobrachium*. In Proceedings of the 3rd Brazilian Symposium on Shrimp Culture. Joao Pessoa, Paraiba, Brazil, 1991b; 409-419.
8. Raaijmakers J, Weller DM, Thomashow LS. Frequency of antibiotic-producing *Pseudomonas spp*. in natural environments. Appl Environ Microbiol. 1997; 63: 881-887.
9. Austin B, Austin DA. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. 4th edn. Chichester: Springer-Praxis; 2007.
10. Ramalingam K, Ramarani S. Effect of *Pseudomonas aeruginosa* on the giant freshwater prawn, *Macrobrachium rosenbergii* - histopathological and electron microscopic study. J Environ Biol. 2007; 28: 627-635.
11. Eissa NME, Abou El-Ghiet EN, Shaheen AA, Abbass A. Characterization of *Pseudomonas* species isolated from Tilapia *Oreochromis niloticus* in Qaroun and Wadi-El-Rayan Lakes, Egypt. Glob Vet. 2010; 5: 116-121.
12. Panda S, Bandyopadhyay PK, Chatterjee SN. Characterization of *Pseudomonas aeruginosa* PB112 (JN996498) isolated from infected *Labeo bata* (Hamilton) by 16S rRNA gene sequence analysis and fatty acid methyl ester (FAME) analysis. Afr J Biotechnol. 2013; 12: 400-405.
13. FAO. Cultured Aquatic Species Information Programme *Macrobrachium rosenbergii* (De Man, 1879). FAO Fisheries and Aquaculture Department. Aquatic Animal Pathogen and Quarantine Information System AAPQIS; 2014.

14. Blunden G. Marine algae as sources of biologically active compounds. Interdis Sci Rev. 1993; 18: 73-80.

15. Kaliaperumal N, Kalimuthu S, Ramalingam JR. Economically Important Seaweeds. Special publication number 62. Central marine fisheries research institute. Indian council of agricultural research. 1995.

16. Rajkumar G, Bhavan PS. Phytochemical characterization of the marine brown alga *Turbinaria ornata.* Res J Chem Environ. 2017; 21: 54-63.

17. Patra JK, Patra AP, Mahapatra NK, Thatoi HN, Das S, Sahu RK, et al. Antimicrobial activity of organic solvent extracts of three marine macroalgae from Chilika Lake, Orissa, India. Mala J Micro. 2009; 5: 128-131.

18. Castell JD, Tiews K. Report of the EIFAC, IUNS and ICES working group on the standardization of methodology in fish nutrition research. Hamburg, Federal Republic of Germany, EIFAC Technical Paper; 1980.

19. AOAC International. Official methods of analysis of AOAC international, 16th edition. Volume 1. 1995.

20. Srinivasan V. Dietary concentrations of iron oxide and magnesium oxide nanoparticles in survival, growth, nutritional and immune enhancements of the prawn *Macrobrachium rosenbergii* post-larvae and disease tolerance against *Pseudomonas aeruginosa* infection. Ph.D., Thesis submitted to Bharathiar University, India; 2016.

21. Roque A, Turnbull JF, Escalante G, Gomez-Gil B, Alday-Sanz MV. Development of a bath challenge for the marine shrimp *Penaeus vannamei* Boone, 1931. Aquaculture. 1998; 169: 283-290.

22. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957; 28: 56-63.

23. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974; 47: 469-474.

24. Sinha AK. Colorimetric assay of catalase. Anal Biochem. 1972; 47: 389-394.

25. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95: 351-358.

26. Lowry OH, Rosenbrough WJ, Fair AL, Randall RJ. Protein measurement with the folinphenol reagent. J Biol Chem. 1951; 193: 265-275.

27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.

28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680-685.

29. Baxter J. Local optima avoidance in depot location. J Oper Res Soc. 1981; 32: 815-819.

30. Blum C, Blesa MJ, Roli A, Sampels M. Hybrid Metaheuristics: An Emerging Approach to Optimization. Studies in Computational Intelligence. Berlin Heidelberg: Springer-Verlag; 2008; 114.

31. Kolanjinathan K, Ganesh P, Govindarajan M. Antibacterial activity of ethanol extracts of seaweeds against fish bacterial pathogens. Eur Rev Med Pharm Sci. 2009; 13: 173-177.

32. Pesando D, Caram B. Screening of marine algae from French Mediterranean coasts for antibacterial and antifungal activity. Bot Mar. 1984; 27: 381-386.

33. Zheng Y, Chen Y, Hai-sheng LU. Screening for antibacterial and antifungal activities in some marine algae from the Fujian coast of China with three different solvents. Chin J Ocean Lim. 2001; 19: 327-331.

34. Thanigaivel S, Natarajan C, Amitava M, Thomas J. Investigation of seaweed extracts as a source of treatment against bacterial fish pathogen. Aquaculture. 2015; 448: 82-86.

35. Pholdaeng, K., Pongsamart, S. Studies on the immunomodulatory effect of polysaccharide gel extracted from *Durio zibethinus* in *Penaeus monodon* shrimp against *Vibrio harveyi* and WSSV. Fish Shellfish Immunol. 2010; 28: 555-561.

36. Holdt SL, Kraan S. Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol. 2011; 23: 543-597.

37. Immanuel G, Vincybai VC, Sivaram V, Palavesam A, Marian MP. Effect of butanolic extracts from terrestrial herbs and seaweeds on the survival, growth and pathogens (*Vibrio parahaemolyticus*) load on shrimp *Penaeus indicus* juveniles. Aquaculture. 2004; 236: 53-65.

38. Manilal A, Selvin J, Sujith S, Panikkar MVN. Evaluation of therapeutic efficacy of Indian green alga, *Acrosiphonia orientalis* (J. Agardh) in the treatment of *vibriosis* in *Penaeus monodon*. Thalassas Int J Mar Sci. 2012; 28: 33-46.

39. Selvin J, Manilal A, Sujith S, Kiran GS, Lipton AP. Efficacy of marine green alga *Ulva fasciata* extract on the management of shrimp bacterial diseases. Lat Am J Aquat Res. 2011; 39: 197-204.

40. Bhavan PS, Geraldine P. Histopathology of the hapatopancrease and gills of the prawn *Macrobrachium malcolmsonii* exposed to endosulfan. Aquatic Toxicol. 2000; 50: 331-339.

41. Poobalane S, Thompson KD, Laszlo A, Verjan N, Han KD, Jeney G, et al. Production and efficacy of an *Aeromonas hydrophila* recombinant S-layer protein vaccine for fish. Vaccine. 2010; 28: 3540-3547.

42. Swain P, Behera T, Mohapatra D, Nanda PK, Nayak SK, Meher PK, et al. Derivation of rough attenuated variants from smooth virulent *Aeromonas hydrophila* and their immunogenicity in fish. Vaccine. 2010; 28, 4626-4631.

43. Somboonwiwat K, Chaikeeratisak V, Ching Wang H, Fang Lo C, Tassanakajon A. Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection. Proteome Sci. 2010; 8: 1-11.

44. Alinejad T, Bin KQ, Vejayan J, Othman RY, Bhassu S. Proteomic analysis of differentially expressed protein in hemocytes of wild giant freshwater prawn *Macrobrachium rosenbergii* infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV). Meta Gene. 2015; 5: 55-67.

45. Solis FJ, Wets RJB. Minimization by Random Search Techniques. Mathem Oper Res. 1981; 6: 19-30.

Cite this article

Rajkumar G, Bhavan PS, Srinivasan V, Asaikutti A, Udayasuriyan R (2017) In vitro and In vivo Antibacterial Activity of Marine Alga Turbinaria ornata against Pseudomonas aeruginosa in the Freshwater Prawn Macrobrachium rosenbergii. JSM Biotechnol Bioeng 4(2): 1080.